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The exosome-binding factors Rrp6 and Rrp47 form a composite surface for recruiting the Mtr4 helicase

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

03 June 2014

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the unusually long duration of the review process in this case. Your manuscript has now been seen by three referees whose comments are shown below.

As you will see from the reports, all three reviewers highlight the interest and timeliness of your findings; however, at the same time they do ask that additional functional data be included to fully support the predictions made from the structural work. In particular, I would ask you to focus your efforts in the following experimental points:

- > Please extend biochemical data to the entire assembled exosome to conclusively support the functional relevance of the observed interactions (Ref#1)
- > Please also strengthen the in vivo relevance for these interactions (ref#2) and clarify the usage of various Rrp6 fragments. On the other hand, please do keep the data addressing mutations outside the Rrp6 interaction surface in the manuscript.
- > We would also strongly encourage you to address the impact of interaction mutants on the turnover of exosome targets (ref#2)

In addition, we have had input from an expert advisor who raised the following points:

- > Mtr4 is required for viability, yet, strains lacking Rrp6 and Rrp47/Lpr1 are viable, suggesting that even in this context Mtr4 can perform part of its activity.
- > Ski2 is related to Mtr4 but lacks the N-terminal interaction region present in the latter. Yet, Ski2 is required for mRNA decay by the exosome in the cytoplasm and the cytoplasmic complex lacks Rrp6 and Rrp47/Lpr1. This suggests that Ski2 interacts with the exosome in a different manner with the exosome core.

-> The authors have to rely on a Rrp47/Lpr1 fusion to detect a clear phenotype, suggesting that steric hindrance (possibly impacting on another interaction site) is required to detect the effect of the reported interaction.

While these additional points are not mandatory for the revised manuscript, I would suggest that you take them into account in the discussion (along with all minor points and suggestions raised by the referees).

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:

http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication and thanks again for your patience here. I look forward to receiving your revised manuscript.

REFEREE REPORTS

Referee #1:

This paper describes the structure of a small fragment of the yeast exosome nuclear catalytic subunit Rrp6 together with a fragment of exosome associated Rrp47 and peptide from Mtr4. The data are supported by biochemical and in vivo experiments. The major finding is that the Mtr4 binds the exosome core indirectly via Rrp6-Rrp47 composite interface while another exosome cofactor Mpp6 interacts with the exosome directly. In vivo experiments suggest that Mtr4 Rrp6-Rrp47 interactions have physiological role but the phenotypes are relatively mild.

This paper provides valuable information for people interested in the exosome biology but does not provide a clue for the role of Rrp47 or Mtr4 in exosome mediated RNA hydrolysis.

Below I describe my major concern regarding this paper:

The Authors reconstructed the entire exosome with Rrp6, Rrp47 and Mtr4 while in vitro activity assays are conducted just for a Rrp6- Rrp47 dimeric sub-complex (Figure E3A). They conclude that "In RNA degradation assays, the nuclease region of Rrp6 (Rrp6122-518) showed activity comparable to that of the Rrp61-518 - Rrp47 Δ C . ". This is not what we can see on the presented gel since actually addition of Rrp47 significantly inhibited the activity against both single stranded and partially double stranded RNA substrates. More importantly it is difficult to rationalize assays on a small sub-complex if entire assembly is available. In order to provide valuable information on putative role of all analyzed interactions it is essential to conduct assays using the entire assembly. Even if the effects of Mtr4 or Rrp47 on Rrp6 and Dis3 activities in the context of the exosome ring will not be very significant it will be very important to actually present such data in this paper.

Minor concerns

- 1) Could the Autrohrs provide the information if the largest reconstructed assemblies presented in the Fig1 does not migrate in the exclusion volume of the gel filtration column? If it is the case other column suitable for larger assemblies should be used. Alternatively ultracentrifugation in the glycerol gradient can be employed
- 2) Since, the importance of Rrp6 for the Mtr4 - exosome interactions has already been shown in human system (Lubas et al. 2011) such information should be stated in the Introduction.

Referee #2:

In this manuscript Schuch et al. describe the architecture of the interactions between Rrp6, Rrp47, Mtr4 and the exosome. They use recombinant proteins and gel filtration approaches to define the direct contacts between these factors. They show that the dimer formed between the N-ter domains of Rrp6 and Rrp47 creates an interaction surface for Mtr4, which contributes to the recruitment of the latter protein to the exosome. The authors provide the crystal structure at 2.6 Å resolution of the ternary complex formed by the interaction domains of Rrp6, Rrp47 and Mtr4. Based on the structure, they generate mutants that prevent the interaction between Rrp6/47 and Mtr4 and affect to some extent RNA degradation by Rrp6/exosome. They also demonstrated by mutation that a few conserved residues in the same Rrp6/47 interaction domain (but that are not involved in the interaction with Mtr4) are important for full exosome activity.

This is an excellent paper, presenting new and interesting results on the architecture of the exosome and Mtr4. The quality of the work is high and the paper should be of interest for the wide audience of the journal. The manuscript is clearly written. I would certainly recommend publication in EMBO J. The weak part of this work concerns the *in vivo* validation of the mutants designed based on the structure. At least some of the following points should be addressed before publication.

Specific points:

- in p. 11 the authors hypothesized that the Rrp6/47 interaction surface might serve as an RNA binding site and assessed RNA binding and RNA degradation in the presence or absence of Rrp47. However they did not use the same fragment of Rrp6 when assessing the contribution of Rrp47 (i.e. they compared Rrp6¹²²⁻⁵¹⁸ and Rrp6⁶¹⁻⁵¹⁸-Rrp47). With these data in hand it is not possible to draw definitive conclusions, because differences seen could be due to differences in Rrp6 and not to the presence of Rrp47. This assay should be performed with the same Rrp6 fragments to be conclusive. In the same section the authors show that the C-terminus of Rrp47 increases the affinity for the RNA by one order of magnitude, although this does not significantly influence degradation. In this assay they compare the same fragments of Rrp6, with and without the C-ter of Rrp47, which is correct. However, this experiment appears to address a different point (the role of the C-ter of Rrp47), which is a bit distracting. The authors should re-organize this section.
- The effects observed for the rrp6 I14L,R18E interaction mutant are weak (Fig. 5), the bands should be quantified.
- The authors analyze rrp6 mutants in conserved positions of the interaction surface (e.g. D27,F30 and E90,D97). However these mutations do not affect the interaction with Rrp47 and the reasons why they are conserved is unclear. This part of the work is distracting and I suggest to reduce it to leave some space for some more detailed analysis of the mutations that affect the interaction (see next point).
- The interaction mutants should be analyzed in more detail. The authors show a strong genetic interaction of rrp6 I14L,R18E with mtr4-GFP but whether this is due to a loss of the interaction is not demonstrated. The authors should assess whether *in vivo* the rrp6 I14L,R18E mutation leads to the loss of interaction with Mtr4 and/or of the latter with the exosome. The mtr4-GFP strain might interact even less well than the non-tagged Mtr4 with the rrp6 mutant, a prediction that could be verified by expressing a tagged rrp6 mutant on a wt RRP6 background.
- It would also be interesting to assess the effect of the mtr4-GFP/ rrp6 I14L,R18E combined mutant on degradation of exosome targets. The mutant is not viable, but a strain containing a wild type gene under control of a repressible promoter could be employed.
- Finally the authors should assess the impact *in vivo* of mutants in Mtr4 that affect the interaction with the dimer *in vitro* (mtr4_F7A,F10A, p11). The authors could look at the interaction with the Rrp6/47 dimer and/or the exosome *in vivo*, and eventually at the effects on degradation of exosome targets.

Minor points:

- Fig. 3C: the double mutant I14L,R18E is labeled R18E in the figure, but discussed as a double mutant in the text (p.11), where the panel is referred to as 3B instead of 3C.
- p. 11: it should be stated that the RNA binding assays were performed with the Rrp6 catalytic mutant (inferred from fig. E3)

Referee #3:

Schuch et al. describe biochemical and structural studies of a complex comprising parts of the yeast proteins Rrp6, Rrp47 and Mtr4, which are nuclear-specific cofactors of the exosome. They performed detailed interaction profiling, mapped interacting regions of the proteins, determined binary and ternary complex crystal structures, showed by site-directed mutagenesis that certain residues are crucial for complex stability and revealed *in vivo* defects and genetic interactions due to proteins bearing the same mutations. They summarize their results in a model of the nuclear exosome holoenzyme.

The manuscript is well written, the experimental work appears technically sound and the structures are of high quality. Biochemical and structural findings are linked to *in vivo* function. The structures, showing a remarkably intertwined Rrp6-Rrp47 complex that synergistically binds Mtr4, are novel and interesting. The results are of fundamental importance for our understanding of a complex molecular machine, the exosome, which is involved in many aspects of RNA metabolism. As such, the work should be of interest to a broad molecular biological audience.

Minor points:

1. The authors could perhaps describe in a bit more detail how merohedral twinning in the Rrp6-Rrp47-Mtr4 crystals was dealt with during structure solution and refinement.
2. The rationale for the conducted RNA binding studies (pg. 11, second half) was not clear to this referee. Did the authors think RNA could bind Rrp6-Rrp47 instead of Mtr4? Perhaps competition experiments would have been useful then.
3. The authors could perhaps make a somewhat stronger point of the synergism of Rrp6 and Rrp47 in Mtr4 binding. The structure nicely explains their interaction studies, i.e. the highly intertwined Rrp6-Rrp47 structure generates a binding groove for Mtr4 that is compositely formed by the two proteins, explaining why neither of them binds Mtr4 alone.

1st Revision - authors' response

08 August 2014

Referee #1:

The Authors reconstructed the entire exosome with Rrp6, Rrp47 and Mtr4 while *in vitro* activity assays are conducted just for a Rrp6- Rrp47 dimeric sub-complex (Figure E3A). They conclude that "In RNA degradation assays, the nuclease region of Rrp6 (Rrp6122-518) showed activity comparable to that of the Rrp61-518 - Rrp47 Δ C." This is not what we can see on the presented gel since actually addition of Rrp47 significantly inhibited the activity against both single stranded and partially double stranded RNA substrates.

We agree with the Reviewer. On page 12, we have changed the sentence referring to this Figure to:

"In RNA degradation assays, the Rrp61-518 – Rrp47 Δ C complex showed somewhat lower activity as compared to that of Rrp6122-518 (Fig 6C), indicating that the Rrp6NRrp47N module subtly downregulates the enzymatic properties of the Rrp6 ribonuclease. Although the rationale for this effect is currently unclear, similar observations have been recently reported (Dedic et al., 2014, Barbosa et al., 2014)." We have moved the degradation assays in this Figure and the accompanying RNA binding data from Supplementary to the main text (Fig 6B and 6C of the revised manuscript).

More importantly it is difficult to rationalize assays on a small sub-complex if entire assembly is available. In order to provide valuable information on putative role of all analyzed interactions it is essential to conduct assays using the entire assembly. Even if the effects of Mtr4 or Rrp47 on Rrp6 and Dis3 activities in the context of the

exosome ring will not be very significant it will be very important to actually present such data in this paper.

We have carried out a set of degradation assays on the 14-subunit nuclear exosome complex and its separate components, which include two nuclease activities (Rrp6 and Rrp44) and an ATPase activity (Mtr4). The experiments are shown in **new Fig E3B**, and are discussed on page 12 as follows:

“We carried out a set of degradation assays of Rrp6 Δ NLS – Rrp47 Δ C in the presence of Mtr4, with and without the other subunits of the nuclear exosome holocomplex (Fig E3B). We first tested a double-stranded substrate with a short 3' overhang (10 nucleotides) that from previous work is known to be inaccessible by the Rrp44 exoribonuclease when in the context of Exo-9 (Bonneau *et al*, 2009) (Fig E3B, upper panel). We also tested a double-stranded substrate with a long 3' overhang (35 nucleotides) that is accessible by the processive exoribonuclease activity of Rrp44 (Fig E3, lower panel). We found that the Rrp6-Rrp47 degradation properties on these substrates were not affected by the presence of Mtr4 (Fig E3B). Although we saw no significant effect of Mtr4 on the degradation of these substrates by either Rrp6 or Rrp44, we caution that it is possible that the helicase domain of Mtr4 might operate in the context of more complex RNA structures.”

We point out that understanding how the helicase domain of Mtr4 functions in the context of the exosome complex is an important task for the future that will likely engage us and others for the new few years.

Minor concerns

1) Could the Authors provide the information if the largest reconstructed assemblies presented in the Fig1 does not migrate in the exclusion volume of the gel filtration column? If it is the case other column suitable for larger assemblies should be used. Alternatively ultracentrifugation in the glycerol gradient can be employed. The exclusion volume of the column we used is 0.8 ml (now reported in the legend of Fig 1A). Other methods (such as the use of a Superose6 column for example) did not have the resolution required for these experiments. We note that the elution volume for the largest assembly we tested is 1.01 ml (see Table E1). In addition, if a component of these assemblies would not co-elute together with the other subunits, we would observe a second peak at larger volumes corresponding to the unbound subunit (see for example when Mtr4 is not associated with an exosome complex that lacks Rrp6N-Rrp47N, Fig 1C, peak 6 or Fig 1D peaks 3 and 5).

2) Since, the importance of Rrp6 for the Mtr4 - exosome interactions has already been shown in human system (Lubas *et al*. 2011) such information should be stated in the Introduction.

We have added the reference to the work of Andrzej Dziembowski and Torben Heick Jensen labs.

Referee #2:

At least some of the following points should be addressed before publication.

Specific points:

- in p. 11 the authors hypothesized that the Rrp6/47 interaction surface might serve as an RNA binding site and assessed RNA binding and RNA degradation in the presence or absence of Rrp47. However they did not use the same fragment of Rrp6 when assessing the contribution of Rrp47 (i.e. they compared Rrp6122-518 and Rrp61-518-Rrp47). With these data in hand it is not possible to draw definitive conclusions, because differences seen could be due to differences in Rrp6 and not to the presence of Rrp47. This assay should be performed with the same Rrp6 fragments to be conclusive. In the same section the authors show that the C-terminus of Rrp47 increases the affinity for the RNA by one order of magnitude, although this does not significantly influence degradation. In this assays they compare the same fragments of Rrp6, with and without the C-ter of Rrp47, which is correct. However, this

experiment appears to address a different point (the role of the C-ter of Rrp47), which is a bit distracting. The authors should re-organize this section.

We had actually used these fragments on purpose, based on the structural information, but had not explained the rationale in the submitted version. From the structure, the N-terminal domains of Rrp6 and Rrp47 form a highly intertwined structure: they cannot be properly folded in isolation. We on purpose avoided carrying out quantitative binding experiments with partially unfolded proteins. We now explain the rationale on page 11:

“Since from the structure the N-terminal region of Rrp6 is expected to be unfolded in the absence of Rrp47, we compared Rrp61-518 – Rrp47 Δ C with Rrp6122-518.”

As suggested, we now removed the analysis of the influence of the C-terminus of Rrp47 on RNA binding and degradation.

- The effects observed for the *rrp6* I14L,R18E interaction mutant are weak (Fig. 5), the bands should be quantified.

Quantification of the major bands referred to in the text (5.8S+30, the 5' ETS fragment and the U3 and 5S rRNA degradation fragments) is presented in the revised Figure (**new Fig 7**). Although the 5.8S rRNA processing phenotype of the I14E R18E mutant is considerably weaker than the D27R F30R mutant, it was nevertheless consistently observed in both *rrp6 Δ* and in *rrp47-zz rrp6 Δ* strains. We have also included additional data from northern analyses of the *rrp6 Δ* mutant in the revised manuscript to support this (**new Fig E4A**). We now specify on page 13:

“The phenotype was stronger for the *rrp6*D27R,F30R mutant and weaker with the *rrp6*I14E,R18E mutant, but reproducible.”

- The authors analyze *rrp6* mutants in conserved positions of the interaction surface (e.g. D27,F30 and E90,D97). However these mutations do not affect the interaction with Rrp47 and the reasons why they are conserved is unclear. This part of the work is distracting and I suggest to reduce it to leave some space for some more detailed analysis of the mutations that affect the interaction (see next point).

We have reduced the discussion on these data (specifically we have condensed the last Results paragraph in the original version) and placed more emphasis on the analysis of the mutations that affect the interaction (next point).

However, on the request of the editor, we have left the data on these mutants in Fig 7 and a brief discussion. On page 14, we now state:

“It is currently unclear why conserved surface residues of Rrp6-Rrp47 that are not involved in Mtr4N binding are important for function, but it is possible that they are involved in additional interactions within the nuclear exosome complex or with the substrate ribonucleoprotein particle.”

- The interaction mutants should be analyzed in more detail. The authors show a strong genetic interaction of *rrp6* I14L,R18E with *mtr4*-GFP but whether this is due to a loss of the interaction is not demonstrated. The authors should assess whether *in vivo* the *rrp6* I14L,R18E mutation leads to the loss of interaction with Mtr4 and/or of the latter with the exosome. The *mtr4*-GFP strain might interact even less well than the non-tagged Mtr4 with the *rrp6* mutant, a prediction that could be verified by expressing a tagged *rrp6* mutant on a wt RRP6 background.

We have included these important analyses on the Mtr4/Rrp6 interaction in yeast in the revised manuscript (**new Fig 8B**). The key finding is that the *rrp6* I14E R18E mutation disrupts the interaction between Rrp6 and Mtr4 in yeast.

- It would also be interesting to assess the effect of the *mtr4*-GFP/ *rrp6* I14L,R18E combined mutant on degradation of exosome targets. The mutant is not viable, but a strain containing a wild type gene under control of a repressible promoter could be employed.

We agree that it would be interesting to assess the impact of the Rrp6 and Mtr4 mutants also on the turnover of exosome targets, but we believe that this goes beyond the scope of this manuscript, which already contains a large set of structural, biophysical, biochemical and *in vivo* data. The inclusion of the northern blot data from the *mtr4-gfp rrp6* D27RF30R and *mtr4-gfp rrp6* E90R E97R mutants does

however provide some indication of the synergistic effects of *mtr4-gfp rrp6* mutants.

- Finally the authors should assess the impact in vivo of mutants in Mtr4 that affect the interaction with the dimer in vitro (*mtr4_F7A,F10A*, p11). The authors could look at the interaction with the Rrp6/47 dimer and/or the exosome in vivo, and eventually at the effects on degradation of exosome targets.

In the revised manuscript we have included pull-down assays showing that the *mtr4* F7A F10A mutant disrupts the interaction between Mtr4 and Rrp6 (**new Fig 8B**). Furthermore, we have included additional genetic data showing that the combination of the F7A F10A mutation and the GFP C-terminal fusion of Mtr4 causes loss of function *in vivo* (**new Fig 8C**). These data reinforce our conclusion that the Mtr4/Rrp6 interaction described in this manuscript is critical. We attempted to analyze interactions between Mtr4-gfp and the exosome core but were unsuccessful, probably because the GFP tag impacts on the Mtr4/exosome interaction.

Minor points:

- Fig. 3C: the double mutant I14L,R18E is labeled R18E in the figure, but discussed as a double mutant in the text (p.11), where the panel is referred to as 3B instead of 3C.

Corrected.

- p. 11: it should be stated that the RNA binding assays were performed with the Rrp6 catalytic mutant (inferred from fig. E3)

We modified the text on page 11 to:

“We measured RNA-binding affinities by fluorescence anisotropy using fluoresceinlabeled poly(A)₃₅ or poly(U)₃₀ RNAs and using a catalytic mutant of Rrp6 (Asp296 to Asn).”

Referee #3:

Minor points:

1. The authors could perhaps describe in a bit more detail how merohedral twinning in the Rrp6-Rrp47-Mtr4 crystals was dealt with during structure solution and refinement.

We thank the Reviewer for this suggestion: structure determination had indeed been technically challenging because of the twinning problems. We have now added a description of how we dealt with merohedral twinning during structure solution and refinement. On page 21 we state:

“Merohedral twinning generated by a two-fold axis perpendicular to a crystallographic 3-fold axis of the trigonal space group became apparent by the poor quality of the electron density for one of the three copies of the complex in the asymmetric unit. The twin law (-h,-k,l) and twinning fraction (0.5, perfect twin) was determined using phenix.xtriage (Adams *et al*, 2010). After manual tracing of the Mtr4N sequence, the model was completed using Coot and refined against twinned data using phenix.refine.”

2. The rationale for the conducted RNA binding studies (pg. 11, second half) was not clear to this referee. Did the authors think RNA could bind Rrp6-Rrp47 instead of Mtr4? Perhaps competition experiments would have been useful then.

From the structure, we believe this surface binds Mtr4. However, we felt we needed to validate this because Rrp47 has been shown to bind RNA and because the surface is positively charged. On page 11 we state:

„The concave surface of Rrp6N – Rrp47N that binds Mtr4N is highly positively charged (Fig. 6A). Given these electrostatic properties and previous reports that Rrp47 binds RNA (Stead *et al*, 2007), we tested whether the interaction between Rrp6 and Rrp47 might also serve as an RNA-binding site (at least in the absence of Mtr4).“

3. The authors could perhaps make a somewhat stronger point of the synergism of

Rrp6 and Rrp47 in Mtr4 binding. The structure nicely explains their interaction studies, i.e. the highly intertwined Rrp6-Rrp47 structure generates a binding groove for Mtr4 that is compositely formed by the two proteins, explaining why neither of them binds Mtr4 alone.

We thank the Reviewer for pointing this out. We have changed the abstract accordingly.

Referee/Advisor #4:

Mtr4 is required for viability, yet, strains lacking Rrp6 and Rrp47/Lpr1 are viable, suggesting that even in this context Mtr4 can perform part of its activity.

On pages 17/18, we added:

“The presence of additional weak interactions would also rationalize why Mtr4 would be able to carry out at least part of its functions *in vivo* in the absence of Rrp6 and Rrp47, as can be inferred from the severity of the corresponding knock-out studies (la Cruz *et al*, 1998; Briggs *et al*, 1998; Mitchell *et al*, 2003).”

Ski2 is related to Mtr4 but lacks the N-terminal interaction region present in the latter. Yet, Ski2 is required for mRNA decay by the exosome in the cytoplasm and the cytoplasmic complex lacks Rrp6 and Rrp47/Lpr1. This suggests that Ski2 interacts with the exosome in a different manner with the exosome core.

On page 8, we added:

“As a note, the cytoplasmic Ski2 helicase does not contain an analogous N-terminal sequence, and consistently the cytoplasmic exosome complex does not contain Rrp6 and Rrp47.”

-> *The authors have to rely on a Rrp47/Lpr1 fusion to detect a clear phenotype, suggesting that steric hindrance (possibly impacting on another interaction site) is required to detect the effect of the reported interaction.*

The RNA phenotypes shown for the *rrp6* mutants in Fig 7 are not dependent upon expression of the Rrp47-zz fusion protein. We have datasets on *rrp6Δ* and *rrp47-zz rrp6Δ* strains using plasmids expressing zz-Rrp6 fusion proteins or non-tagged Rrp6 proteins, all of which showed essentially the same phenotypes but to differing degrees of intensity. We included the analysis of the *rrp47-zz* strain in Fig 7 because this allowed us to present a complete dataset including important control western analyses on the Rrp47 protein (**new Fig 7C**). We have now included additional data in the revised manuscript (**new Fig E4A**) showing that the *rrp6* mutant phenotype is observed independent of the *rrp47-zz* allele.

Accepted

26 August 2014

Thank you for submitting your revised manuscript for The EMBO Journal. Your study has now been seen by two of the original referees (comments included below) and as you will see they both find that all criticisms have been addressed. I am therefore happy to inform you that your manuscript has been accepted for publication with us.

If you have any questions, please do not hesitate to contact me. Thank you for your contribution to The EMBO Journal and congratulations on this nicely executed work!

REFEREE REPORTS

Referee #1:

The authors made a substantial effort to address all my comments as well as comments of other reviewers. Overall, the manuscript is improved significantly and I can recommend it for publication.

Referee #2:

The authors have responded successfully to the minor points I had raised. The manuscript is now suited for publication in EMBO Journal